

Phylogenetic studies of four species of ciliate inferred from 16S-like small subunit rRNA gene sequences

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Abstract: The phylogenetic relationships of four ciliate genera (*Urostyla*, *Euplotes*, *Stylonychia* and *Pseudokeronopsis*), which also are the important environment inspection species, were analyzed by the comparison of small subunit ribosomal RNA gene sequences. *Euplotes* appeared as an early branching group whose divergence from the hypotrichous line at a deep level was strongly supported by parsimony and matrix analyses. The analyses also supported the hypothesis that there were closely relationship between species in *Urostyla* and *Holosticha*. The sibling species *Stylonychia mytilus* and *S. lemnae* could be separated by the evolutionary analyses. Furthermore, *Pseudokeronopsis rubra* had relatively more closely relationship with the species in Holostichidae than that in Urostylidae based on the evolutionary distance value.

Keywords: *Euplotes encysticus*; phylogenetic relationship; *Pseudokeronopsis rubra*; SS rRNA gene; *Stylonychia mytilus*; *Urostyla grandis*

Introduction

There are many ciliates on the earth, which distribute in soil, swamp, lake, spring, etc.. The ciliate, as the most preliminary consumer in the ecosystem, plays an important role in material and energy recycle, as well as in environment inspection. Some important kinds of carbide and mineral, which could not be directly used by the foliage, could be transformed to absorbable nourishment by their direct or indirect metabolism. The ciliate community is regarded as one of the important indication factors to the environment inspection and forest breeding because they are very sensitive to the environment variation..

The phylum Ciliophora is a large group of protozoan and comprises at least nine lineages, which are supposed to be monophyletic and have been given class status (de Puytorac et al. 1993; Small et al. 1981, 1985). However, the phylogenetic relationships among some species remain to be discussed, such as the relationship between *Urostyla* and *Holosticha* (Foissner 2004; Hewitt et al. 2003). Molecular characters (gene sequences) provides a new database to test phylogenetic hypotheses that are derived from morphological observations. The blast result of the

sequences of the small and large subunit ribosomal RNA (SS rRNA, LS rRNA) genes has confirmed some of the phylogenetic hypotheses, which are derived from morphological observations and refuted others (e.g. Lynn et al. 2000; Stechmann et al. 1998; Struder-Kypke et al. 2000; Shang et al. 2003). In this study we focused on the phylogenetic relationship of some species of stichotrichs and hypotrichs. The SS rRNA gene of *Urostyla grandis*, *Euplotes encysticus*, *Stylonychia mytilus* and *Pseudokeronopsis rubra* were analyzed in order to provide more information of their phylogenetic relationships within the class Spirotrichea.

Materials and methods

Ciliate collection

Urostyla grandis, *Euplotes encysticus* and *Stylonychia mytilus* were sampled from the lake of Changfeng Park, near East China Normal University, Shanghai, China. The subsequent isolation, impregnation, and identification were carried out according to the descriptions by Small et al. (1985), Gu et al. (1990), Hu et al. (2001) and Song et al. (2006). Clonal cultures were established and maintained in filtrated water at room temperature with rice grains as food source to enrich bacteria. *Pseudokeronopsis rubra* (kindly provided by Prof. Weibo Song, Ocean University of China) was maintained in filtrated seawater.

DNA extraction

The DNA extraction method was modified with the reference of that reported by Sambrook (1992). Cells were rinsed three times with filtrated water after being starved overnight and were then pelleted by centrifugation. Pelleted cells were dissolved

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respectively in two kinds of solution, firstly in 2-mL solution (0.02M EDTA, 0.05M Tris), and afterwards 20 μ L of 100- μ g-mL⁻¹ Proteinase K, secondly in 2-mL solution (0.02-M EDTA, 0.05M Tris, 2%SDS). After incubated at 56°C for 24h, 5-M NaCl was added to the solution for 20 min with ice incubation to remove the protein. DNA was precipitated with 70% ethanol and stored at -20°C.

PCR amplifying and sequencing

A total volume of 50- μ L mixture was used to amplify the SS rRNA gene, including 25 ng of genomic DNA, 5-U Taq Polymerase (Takara Bio. Co., Japan), 10-mM Tris-HCl (pH 8.3), 50-mM KCl, 1.5-mM MgCl₂, 10-mM dNTP, oligonucleotide primer (4.15- μ M 16s-like F: 5'-AACCTGGTTGATCCTGCCAGT-3' and 4.73- μ M 16s-like R: 5'-TGATCCTTCTGCAGGTTACCTAC-3') designed by Medlin et al. (1988). The protocol used to obtain PCR products was as follows: the reaction mixtures were firstly denatured at 94°C for 3 min; followed by 36 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; and a final extension step at 72°C for 10 min.

The PCR products were purified and sequenced in two directions with the ABI Prism 377-18 Automated DNA Sequencer (Huada Bio. Co.). All sequences were confirmed from both strands.

Sequence availability

The nucleotide sequences used in this paper are available from the GenBank databases under the following accession numbers: *Stylonychia pustulata* (M14600), *Pleurotricha lanceolata* (AF164128), *Gastrostyla steinei* (AF164133), *Uroleptus pisces* (AF164131), *Paruroleptus lepisma* (AF164132), *Uroleptus gallina* (AF164130), *Engelmanniella mobilis* (AF164134), *Onychodromus grandis* (AJ310486), *Steinia sphagnicola* (AJ310494), *Pattersoniella vitiphila* (AJ310495), *Laurentiella strenua* (AJ310487), *Gonostomus strenuum* (AJ310493), *Holosticha multistylata* (AJ277876), *Sterkiella nova* (X03948), *Paraurostyla weissei* (AF164127), *Euplotes aediculatus* (AF164136), *Euplotes crassus* (AJ310492), *Orthoamphisiella breviseries* (AY498654), *Stylonychia lemnae* (AF164124), *Oxytricha longa* (AF164125), *Hemiurosoma terricola* (AY498651), *Onychodromopsis flexilis* (AY498652), *Gonostomum namibiense* (AY498655), *Cyrtohymena citrine* (AY498653), *Oxytricha nova* (M14601), *Uronychia transfuga* (AF260120), *Diophrys appendiculata* (AY004773), *Aspidisca steini* (AF305625), *Onychodromus quadricornutus* (X53485), *Favella panamensis* (AY143572), *Eutintinnus pectinis* (AY143570), *Strombidium purpureum* (U97112) and *Euplotoides vannus* (AJ310488).

Phylogenetic analyses

The sequences were aligned with other SSrRNA gene sequences and the procedures used Clustal X, ver. 1.8 (Thompson et al.

1997) with default parameters and refined by eye. The complete alignment was used for different phylogenetic analyses. Mega, ver. 2.0 was used to calculate the similarity and revolutionary distance between pairs of nucleotide sequences with the two-parameter model of Kimura (1980), using a transition/transversion ratio of 2.0. Neighbor-joining (NJ) distance trees (Saitou et al. 1987), were generated with Mega ver. 2.0 (bootstrap resampled 10,000 times), and maximum-likelihood analysis with the software PAUP 4.0, (bootstrap resampled 100 times) for the ML trees. *Blepharisma americanum* (GenBank Accession No. M97909) was selected as the outgroup taxon for all analyses of the nuclear DNA gene. *Blepharisma* was distantly related to the selected protozoan based on a variety of taxonomic criteria.

Results

Sequences and comparisons

The SS rRNA gene sequences were determined for *Urostyla grandis* (1660 nucleotides, GenBank accession number EF535731), *Euplotes encysticus* (1669 nucleotides, GenBank accession number EF535729), *Stylonychia mytilus* (1667 nucleotides, GenBank accession number EF535730), *Pseudokeronopsis rubra* (1665 nucleotides, GenBank accession number EF535729). The GC content (44.0% *Urostyla grandis*; 43.6% *Euplotes encysticus*; 45.3% *Stylonychia mytilus*; 44.7% *Pseudokeronopsis rubra*) was in the same range as most other ciliates (Elwood et al. 1985; Sogin et al. 1986; Schlegel et al. 1991). The sequences of *Urostyla grandis*, *Stylonychia mytilus*, and *Pseudokeronopsis rubra* were similar to each other, and the sequence of *Euplotes encysticus* differed in 148 nucleotides from that of *Stylonychia mytilus*.

The evolutionary distance value was calculated pairwise between the sequences aligned and those of other species as well as *Blepharisma americanum* (Table 1). From these data it can be seen that the evolutionary distance value for *Stylonychia lemnae* and *Stylonychia mytilus* is only 0.017, suggesting that these two species are closely related, while the most closely related species to *Urostyla grandis* is the most closely related species to *Holosticha multistylata* (evolutionary distance value 0.048). *Euplotes crassus* and *Euplotoides vannus*, which are members of the same clade, have more close relationship with *Euplotes encysticus* (evolutionary distance values 0.053 and 0.054, respectively) than with *Euplotes aediculatus* (0.077). The distance value for *Pseudokeronopsis rubra* and *Urostyla grandis*, *Stylonychia mytilus*, *Holosticha multistylata* is 0.089, 0.084, 0.082, respectively.

Distance matrix analysis

Neighbor-joining (NJ) analyses give strong bootstrap support for the monophyly of the class Stichotrichia (97% NJ, Figs 1, 2), showing Heterotrichea to be the sister taxon to this lineage. The monophyly of the class Choreotrichia is also well supported (99% NJ). According to these analyses, the class Oligotrichia is

probably paraphyletic (Figs 1, 2), whereas, the sister group relationship between Hypotrichia (e. g. *Uronychia transfuga*, *Diophrys appendiculata*, *Aspidisca steini*, *Euplotes aediculatus*,

Euplotes encysticus, *Euplotes crassus*, *Euplotoides vannus*) and Stichotrichia is not bootstrap supported.

Table 1. Kimura 2-parameter evolutionary distance (lower half) and standard errors (upper half)

Species	<i>Holosticha multistylata</i>	<i>Urostyla grandis</i>	<i>Pseudokeronopsis rubra</i>	<i>Stylonychia lemnae</i>	<i>Stylonychia mytilus</i>	<i>Euplotes crassus</i>	<i>Euplotoides vannus</i>	<i>Euplotes encysticus</i>	<i>Euplotes aediculatus</i>
<i>Holosticha multistylata</i>		0.005	0.007	0.005	0.006	0.011	0.011	0.012	0.011
<i>Urostyla grandis</i>	0.048		0.008	0.007	0.007	0.012	0.012	0.012	0.012
<i>Pseudokeronopsis rubra</i>	0.082	0.089		0.008	0.007	0.013	0.013	0.012	0.013
<i>Stylonychia lemnae</i>	0.047	0.069	0.089		0.003	0.011	0.011	0.011	0.011
<i>Stylonychia mytilus</i>	0.053	0.071	0.084	0.017		0.011	0.011	0.012	0.012
<i>Euplotes crassus</i>	0.173	0.192	0.205	0.170	0.180		0.001	0.006	0.007
<i>Euplotoides vannus</i>	0.175	0.193	0.206	0.172	0.182	0.002		0.006	0.007
<i>Euplotes encysticus</i>	0.185	0.186	0.199	0.179	0.181	0.053	0.054		0.007
<i>Euplotes aediculatus</i>	0.181	0.201	0.210	0.175	0.187	0.077	0.078	0.077	

As shown in Fig. 1, *Stylonychia mytilus* forms a clade with *Stylonychia lemnae* and *Laurentiella strenua* (93% NJ), which is a sister group to a lineage that including *Steinia sphagnicola*, *Pattersoniella vitiphila*, *Gastrostyla steinii*, *Onychodromus quadricornutus*, *Pleurotricha lanceolata*, *Onychodromus grandis*, *Stylonychia pustulata*, *Oxytricha nova* and *Sterkiella nova*. *Urostyla grandis* and *Holosticha multistylata* also form a monophyly with strong bootstrap support (91% NJ, 67% ML). Within the class Stichotrichia, the Pseudokeronopsidae branches basally with strong bootstrap support (97% NJ). The major aspects of the topology of the maximum-likelihood trees (Fig. 2) are similar to those of the distance matrix trees (Fig. 1).

Discussion

Based on the analysis of the SS rRNA gene sequences, *Euplotes* appears an early branching lineage with high evolutionary rate, which is consistent with other molecular studies (Chen 2002; Kyoou et al. 2000; Petroni 2002). Moreover, *Euplotes* exhibits some molecular and morphological (Curds 1975) traits, such as a high evolutionary rate in elongation factor 1 α (Moreira et al. 1999), Myb Genes with Myb-2R domains (Yang et al. 2003), three characteristic cirral groups (nine FVC, five transversal cirri, and three caudal cirri), (Schwarz 2007), and cysts form in the lifecycle, which could also be used in phylogenetic analyses (Bussers et al. 1974; Reid et al. 1983; Walker et al. 1980). On the basis of our results, the species in the class of Hypotrichia, the clade which include *Aspidisca steini*, *Euplotes aediculatus*, *Euplotes encysticus*, *Euplotes crassus*, and *Euplotoides vannus*, cluster together and diverge firstly from the root, followed by *Diophrys appendiculata* and *Uronychia transfuga*. The evolutionary distance between *Diophrys appendiculata*, *Uronychia transfuga*, and *Aspidisca steini* is 0.094, 0.126, respectively. Our results also confirmed that *Diophrys appendiculata* and *Uronychia transfuga* are more closely related to each other than to other species in the Hypotrichia, as has been proved by related study (Li et al. 2006). The branch length varying greatly in the Hypotrichia attracted much attention. Many explanations have been postulated (Dini et al. 1999;

Philippe 2000; Petroni 2002), due to its abundance and its ubiquitous distribution. And more extensively studies are needed to reveal their phylogenetic relationship.

The phylogenetic position of the genus *Stylonychia* has been proved (Ammermann et al. 1983; Berger et al. 1997; Bernhard et al. 2001; Wirnsberger et al. 1986), which has been classified as a member of the family Oxytrichidae (Lynn 2003). Although the sibling species, including *Stylonychia mytilus* and *S. lemnae*, could hardly be distinguished by morphological features, they could be separated on the bases of evolutionary analyses. Their presence in the same clade is moderately supported [75% (ML), 91% (NJ)], therefore SS rRNA gene comparison analysis is potentially very useful technique for the separation of morphological similar taxa (Petroni et al. 2002; Schlegel et al. 2003).

Some groups of ciliate, including *Holosticha*, *Keronopsis*, *Pseudokeronopsis* and even some different taxa share a similar ciliary pattern, for example, the zig-zag paired midventral rows and the clearly differentiated frontal, frontoterminal and transverse cirri, their phylogenetic relationship and position is one of the most controversial issue (Hewitt 2003). Over 13 species of *Pseudokeronopsis* have been morphologically studied (Borror 1972, 1983; Foissner 1984; Hu et al. 2001). Based on the unified features, that is, the presence of a bicorona and the far posteriorly extending distal end of the adoral zone of membranelles, Borror et al. (1983) united *Pseudokeronopsis* (including *Uroleptopsis* as synonym) and *Thigmokeronopsis* in the Pseudokeronopsidae basically using the system proposed by Wicklow (1981). This modification in taxonomic schemes has been suggested by other studies (Berger 2004; Eigner and Foissner 1992). Lynn and Corliss (1991) have classified genus *Pseudokeronopsis* as well as *Holosticha* in the Holostichidae, and *Urostyla grandis* in the family Urostylidae (Shi 1999; Shi et al. 1999). However, both *Urostyla* and *Holosticha* have been put in the Urostylidae with the common characteristics including: (i) several larger frontal cirri compared with frontoventral cirri, (ii) frontoventral cirri as a single zig-zag file of paired cirri or a series of shorter files off-set at their anterior and posterior ends (Small et al. 2000).

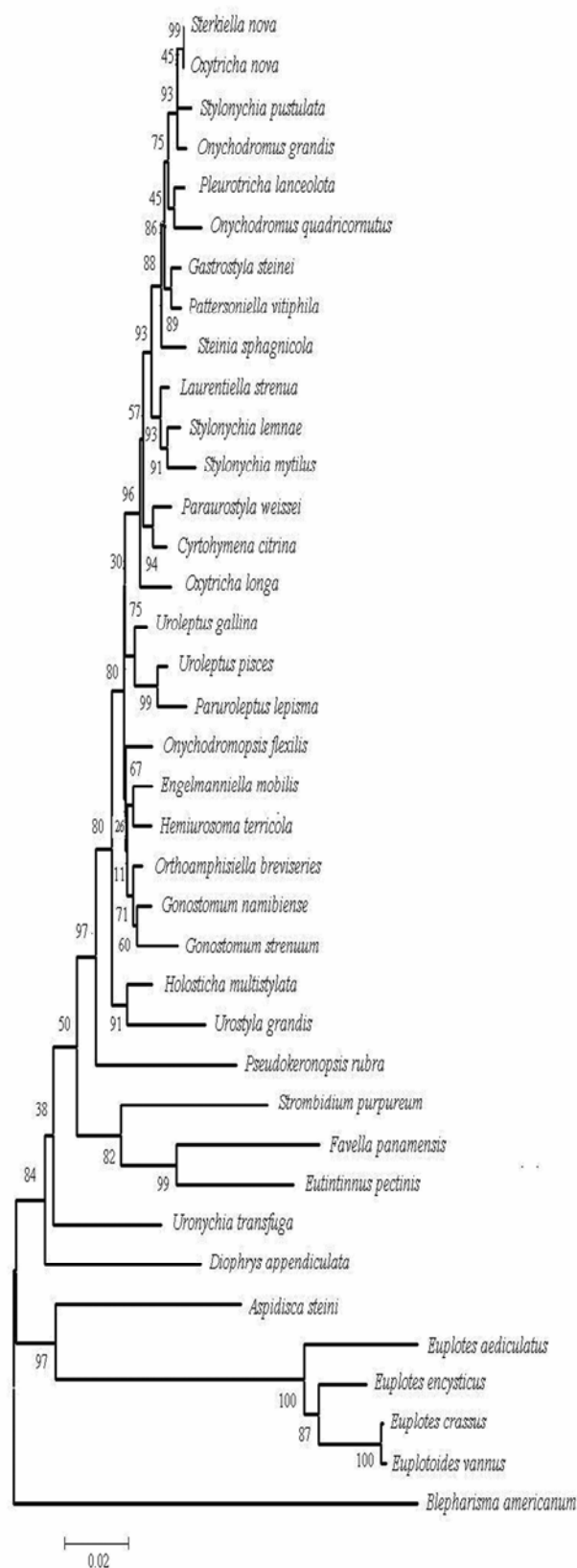


Fig. 1 A distance tree of the ciliate inferred from SS rRNA gene. The number at the nodes represented the bootstrap percentages of 10,000. Evolutionary distance is represented by the length. The scale bar corresponds

to 2 substitutions per 100 nucleotide position.

The nuclear rDNA analysis indicated that a close phylogenetic relationship existed between the last two organisms (Hewitt 2003). In the present study, *Urostyla grandis* and *Holosticha multistylata* cluster together with moderately bootstrap support (91% NJ; 75% ML) (Figs 1, 2). The revolutionary distance between these two species is 0.048 (Table. 1). All these data indicated that *Urostyla grandis* and *Holosticha multistylata* might be phylogenetically closer with each other. And the result agreed to the classification of putting *Urostyla grandis* and *Holosticha multistylata* in the same family (Small et al. 2000). The distance value for *Urostyla grandis* and *Pseudokeronopsis rubra* is 0.089, whereas, *Holosticha multistylata* and *Pseudokeronopsis rubra* is 0.082, which suggests that *Holosticha multistylata*, *Urostyla grandis* and *Pseudokeronopsis rubra* might also be closely related species (67%, 58% ML and 91%, 80% NJ, respectively). Furthermore, *Pseudokeronopsis rubra* is relatively more closely related to the species in Holostichidae.

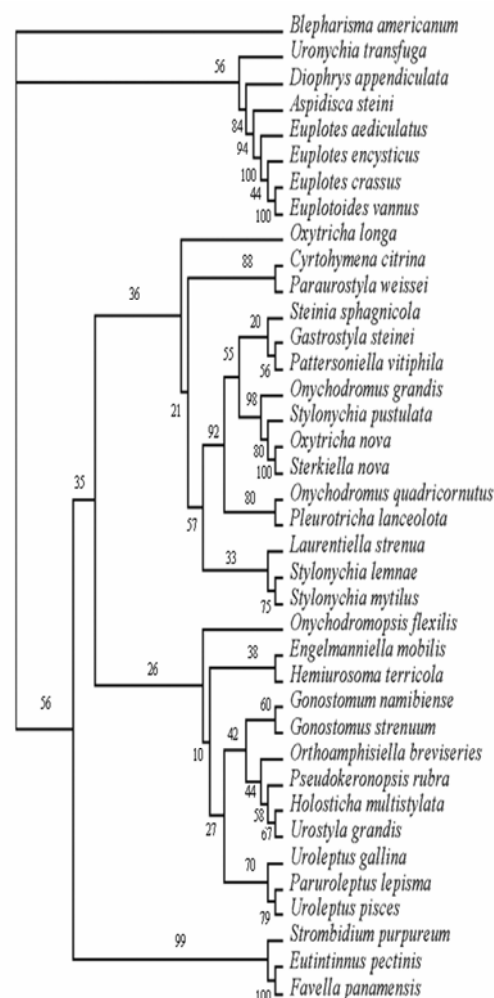


Fig. 2 A maximum-likelihood tree inferred from SS rRNA sequences. The numbers at the nodes represent the real values for the group out of 100 trees in maximum-likelihood method in the PAUP package.

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